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The phylogeny of Galerucinae (Coleoptera: Chrysomelidae) and the performance of mitochondrial genomes in phylogenetic inference compared to nuclear rRNA genes

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Abstract

With efficient sequencing techniques, full mitochondrial genomes are rapidly replacing other widely used markers, including the nuclear rRNA genes, for phylogenetic analysis but their power to resolve deep levels of the tree remains controversial. We studied phylogenetic relationships of leaf beetles (Chrysomelidae) in the tribes Galerucini and Alticini (root worms and flea beetles) based on full mitochondrial genomes (103 newly sequenced), and compared their performance to the widely sequenced nuclear rRNA genes (full 18S, partial 28S). Our results show that: 1) the mitogenome is phylogenetically informative from subtribe to family level, and the per-nucleotide contribution to nodal support is higher than that of rRNA genes; 2) the Galerucini and Alticini are reciprocally monophyletic sister groups, if the classification is adjusted to accommodate several 'problematic genera' that do not fit the dichotomy of lineages based on the presence (Alticini) or absence (Galerucini) of the jumping apparatus; 3) the phylogenetic results suggest a new subtribe system of Galerucini with eight subtribes: Oidina, Galerucina, Hylaspina, Metacyclina, Luperina, Aulacophorina, Diabroticina and Monoleptina.

Keywords: Galerucinae; mitochondrial genomes; phylogenetic informativeness; jumping apparatus; subtribe arrangement.

Introduction

The discipline of phylogenetics attempts to recover evolutionary relationships among taxa as the basis for formal biological classification (Wiley and Lieberman, 2011). Phylogenetic inference relies on a growing number of genes and increasing taxon sampling (Mason-Gamer and Kellogg, 1996; Poe, 1998; Danforth et al., 2005; Nabhan and Sarkar, 2011; Townsend and Leuenberger, 2011; Horreo, 2012) and novel mathematical approaches (Lin et al., 2002; Philippe, 1997). However, the availability of sequence data is still limited and frequently there is a trade-off between the inclusion of many species but few genes, or of few species and many genes (Sanderson et al., 2003; Driskell et al., 2004; Hunt and Vogler, 2008; Song et al., 2010; Liu et al., 2015). The large-scale sequencing of mitochondrial genomes (mitogenomes) may be a compromise for generating sufficient data per taxon while also including numerous exemplars (Timmermans et al., 2016). The effort required for sequencing full mitogenomes has dropped dramatically with the possibility to assemble these sequences from mixtures of specimens that are shotgun sequenced either from long-range PCR products (Timmermans et al., 2010) or directly from total genomic DNA (Gillett et al., 2014; Crampton-Platt et al., 2015; Tang et al., 2015). The approach has been applied to resolve various phylogenetic questions in entomology at taxonomic levels from within families to inter-ordinal relationships (Timmermans et al., 2010; Li et al., 2012; Wan et al., 2012; Gillett et al., 2014; Gomez-Rodriguez et al., 2015; Song et al., 2016; Timmermans et al., 2016).

However, the use of mitogenomes alone poses the risk that the idiosyncrasies of sequence variation in a single marker produce misleading phylogenetic signal, for an incorrect topology supported by a lot of data (Bernt et al., 2013; Simon and Hadrys, 2013). Mitogenome sequencing gained notoriety when deep relationships were apparently recovered incorrectly, which led many to the conclusion that mitogenomes are not useful for resolving deep relationships (Hassanin et al.,

2005; Carapelli et al., 2007; Masta et al., 2009). Sequence variation in mitogenomes (and other markers) generally suffers from saturation, heterogeneity of rates, heterogeneity of nucleotide composition, and the overall complexity of sequence variation that is poorly captured by standard likelihood models, which may produce misleading signal. These issues can potentially be resolved by denser taxon sampling, which reduces long-branch attraction and permits more accurate estimates of (rates of) character change. In addition, more elaborate evolutionary models can improve phylogenetic inferences. For example, (Talavera and Vila, 2011) used 55 insect (Eumetabola) mitogenomes and found long-branch attraction artifacts that were misleading the deep relationship of insects due to saturation of sequence variation and heterogeneity in nucleotide composition. However, these estimates were much by using a site-heterogeneous mixture model (CAT) implemented in the PhyloBayes software (Lartillot et al., 2013). In more recent studies of insect phylogeny at intra- and inter-ordinal levels, compositional heterogeneity was found to be high, but again the CAT model resulted in defensible tree topologies (Timmermans et al., 2015; Song et al., 2016). However, the calculations are computationally demanding and the use of simpler Bayesian models would be desirable as datasets grow.

We studied the Galerucinae, a subfamily of leaf beetles (Coleoptera: Chrysomelidae) to address the power of mitogenomes in phylogenetic inferences, compared with the nuclear ribosomal RNA (rRNA) genes, which arguably are the most widely used markers in molecular phylogenetics. The Galerucinae is the largest subfamily of the Chrysomelidae and includes approximately 14500 described species placed in over 1100 genera occurring worldwide. The Galerucinae has been split into two tribes, the Alticini and Galerucini (Bouchard et al., 2011; Nadein and Bezděk, 2014), which are defined by the presence (Alticini) or absence (Galerucini) of the modified extensor tendon (MET) in the hind femora (also known as metafemoral spring, ~~or~~ metafemoral apodeme, or

Maulik's organ), a structure that permits large jumps for predator evasion and led to their common name of 'flea beetles' (Furth and Suzuki, 1990; Furth and Suzuki, 1998; Nadein and Betz, 2016). However, the placement of several genera within the Alticini-Galerucini dichotomy has been considered to be 'problematic' (Furth and Suzuki, 1994) because the presence or absence of a MET present does not fit with other characters, possibly because the jumping apparatus has arisen multiple times and thus an 'Alticini' defined by the MET becomes polyphyletic (Ge et al., 2011). In addition, given the variability of this trait, it is conceivable that species without MET may be closely related to the jumping Alticini, after the secondary loss of the jumping apparatus.

While progress has been made on the phylogenetics of Alticini, molecular analyses of Galerucini (Galerucinae) remain limited. Starting with (Lingafelter and Konstantinov, 1999), who used nine genera of four tribes to resolve the relationship of Galerucini and Alticini, (Gillespie et al., 2004) greatly increased the taxon sampling for combined data of *cox1*, *18S rRNA* and *28S rRNA-D2*, but basal relationships had generally low support and were sensitive to different analysis methods. (Ge et al., 2011) study of the evolution of the metafemoral spring focused on Alticini but also included 44 taxa of Galerucini as outgroup, showing that some 'problematic genera' previously included in Alticini should be placed with Galerucini. Within Galerucini, currently six subtribes are recognized, including Hylaspina, Oidina and Galerucina that have been recovered as monophyletic, whereas Metacyclina and Luperina were paraphyletic (Gillespie et al., 2004; Ge et al., 2011).

Using mitochondrial genomes and nuclear rRNA genes, we address the following topics. First, we determine the properties of mitochondrial genome sequences to make predictions about their po[illegible] as phylogenetic markers, and test the phylogenetic estimate against the trees from nuclear rRNA genes. Second, we establish the molecular phylogenetic history of Galerucini, to improve on the evolutionary classification. Third, we are interested in the sister groups of the 'problematic

genera' whose placement is not consistent with the presence or absence of the jumping apparatus, for a more refined analysis of multiple origins (Ge et al., 2011) *versus* the secondary loss of a complex trait.

Material and methods

Sampling and sequencing

Samples were collected in the field and preserved in 100% ethanol at -20°C. Voucher specimens for all sampled taxa are kept at the Institute of Zoology, Chinese Academy of Sciences. DNA extraction was from the head and prothorax or the whole body of each specimen depending on their size. Genomic DNA was obtained using a DNeasy Blood and Tissue kit (QIAGEN) and eluted in 200 µL AE buffer and kept at -20°C until used. Six short PCR fragments including the *COI* barcode region (*COI-5'*, amplified with primers HCO/LCO primers), *COI-3'* (amplified using primers Pat/Jerry), *16S rRNA* (*rrnL*), *18S rRNA*, *28S rRNA-D2*, *28S rRNA-D3* were amplified for most specimens. The volume for the PCR reaction was 40 µL including: 26.72 µL ddH_2O, 4 µL $10\times NH_4$ buffer, 1.6 µL 2.5 mM dNTP, 1.6 µL 50 mM $MgCl_2$, 0.08 µL Biotaq DNA polymerase, 2 µL of 10 µM of each primer, and 2 µL DNA template. The PCR conditions involved an initial denaturation step of 5 min at 94ºC; 35 cycles with a denaturation of 30 sec at 94ºC, an annealing step of 50 sec at 51–58ºC (Appendix S1), and an extension step of 60 sec at 72ºC; and a final extension step of 10 min at 72ºC. Primers and their annealing temperature of the PCR are listed in Appendix S1.

PCR fragments were sequenced on both strands. Sequences were assembled using Sequencer 4.8 software (Gene Code Corporation). The six markers were concatenated with Sequence Matrix v.1.7.8 (Vaidya et al., 2011). All newly generated sequences have been deposited

in GenBank under the following accession numbers: KC185460-KC186123; KC255413-KC255499; KU697388- KU697611. Specimens voucher details and accession numbers are given in Appendix S2.

Mitochondrial genomes were sequenced using shotgun sequencing on the Illumina platform. DNA extracts were pooled for library preparation. Two pools were created using the Illumina TruSeq Nano protocol, placing close relatives into different libraries (see (Gillett et al., 2014; Gómez□Rodriguez et al., 2015)). Similar DNA quantities of each extract were included in the pool to minimize undesirable effects of DNA concentration on assembly success. The modal insert size of both libraries was between 600 and 700 bp. Sequencing was performed with the Miseq version 3 kit (2×300 bp paired-end reads) (Illumina Inc., San Diego, CA). Bioinformatics for assembly of mitogenomes followed the pipeline of (Crampton-Platt et al., 2015), with minor modifications. In short, the quality of the raw data was checked using FastQC (Andrews, 2010). Adapters and index motifs were removed using Trimmomatic (Bolger et al., 2014) and subsequently a BLAST search was carried out filtering the data for mitochondrial reads against a custom reference database of 3806 full or partial mitogenome sequences of Coleoptera ($E=1e^{-5}$) with no restriction in length overlap. Low quality reads and short reads (<150 bps) were removed with Prinseq (Schmieder and Edwards, 2011). Genome assembly on the extracted mtDNA reads was performed using IDBA-UD (with minimum k-value 60) (Peng et al., 2012), and the resulting contigs were again filtered for mtDNA hits against the Coleoptera mitogenome database ($E=1e^{-5}$) for sequences with more than 1 kb overlap.

For each contig, tRNA annotations were mapped with COVE (Eddy and Durbin 1994) based on beetle specific tRNA models. The annotated contigs were loaded into Geneious and checked for circularization, followed by the extraction of protein- and rRNA coding genes using

FeatureExtract 1.2 ENRFF_4 (Wernersson, 2005). Annotations were checked against *Diabrotica barberi* (GenBank: NC_022935) as a reference. Finally all protein-coding genes were exported separately, but sequences shorter than 50% of the total gene length were excluded.

Individual contigs were linked to a particular species by matches to the Sanger sequenced *cox1-5'*, *cox1-3'* and *rrnL* used as baits (see Timmermans et al., 2010). A minimum of 98% identity at Blast Alignment was required for a positive identification. In all cases the three baits obtained from a single specimen matched the same contig, indicating the absence of chimeras in the assembly from the mixed shotgun reads (although *rrnL* was missing from a few contigs and thus could not be evaluated in all cases).

Measures of nucleotide variation

Base compositions of mitogenome and nuclear datasets were calculated in MEGA v6.06 (Tamura et al., 2013). The nonsynonymous substitution rate (K_a) among species was calculated with DnaSP v5.0 (Librado and Rozas, 2009). Substitution saturation of different genes was tested in DAMBE5 with the GTR model selected as a reference model (Xia, 2013). The heterogeneity of sequence divergence within datasets relative to an external reference (outgroup) sequence was analyzed with AliGROOVE (Kück et al., 2014) with the default sliding window size. Indels in the nucleotide dataset were treated as ambiguity and the BLOSUM62 matrix was used as default amino acid substitution matrix.

Phylogenetic analysis

Sequences of rRNA genes were aligned separately for each fragment (*18S*, *28S-D2*, *28S-D3*, *rrnL*) with Muscle v.3.8.31 (Edgar, 2004), under default parameters. Protein coding genes (PCGs) were aligned with TransAlign (Bininda-Emonds, 2005). The aligned data from each locus were concatenated with SequenceMatrix v.1.7.8 (Vaidya et al., 2011). Phylogenetic relationships were

inferred from combinations and partitioning of nuclear genes and 13 mitochondrial protein-coding genes, as follows: 1) nuclear genes (119 taxa) partitioned by gene; 2) 13 mitochondrial PCGs (110 taxa) partitioned by gene; 3) 13 mitochondrial PCGs (110 taxa) partitioned by 1st and 2nd codon position and 3rd position removed; 4) amino acid of 13 mitochondrial PCGs (110 taxa) partition by gene; 5) combined nuclear and mitochondrial dataset (118 taxa) partitioned by gene; 6) expanded dataset (273 taxa) using mitochondrial and rRNA data from dataset 1-5 supplemented with GenBank data, partitioned by gene. The first five matrices permitted to test the power of nuclear and mitgenome data available for the same terminals. The expanded matrix broadened the taxon sampling in particular for the (sub) tribes Diabroticina and Metacyclini that were not sampled widely in this study. To be included in the analysis, taxa required a minimum of two genes.

Phylogenetic inferences were performed using MrBayes v.3.2 (Ronquist and Huelsenbeck, 2003), PhyloBayes MPI v1.5a (Lartillot et al., 2013) and TNT (Goloboff et al., 2003; 2008). For MrBayes searches, the most appropriate nucleotide substitution model was selected using the Akaike Information Criterion (AIC) in jModelTest 0.1.1 (Posada, 2008), which was determined for each partition. Model parameters can be found in Appendix S3. The MCMC search was conducted for a minimum of 10,000,000 generations, and sampling was done every 10,000th generations until the average standard deviation of split frequencies was less than 0.01. The first 25% of trees were discarded as "burn-in" and posterior probabilities were estimated for each node. In the PhyloBayes analysis the CAT-GTR model was used for all searches. Two independent tree searches were carried out and stopped after the likelihood of the sampled trees had stabilized and the two runs had satisfactorily converged (maxdiff less than 0.3). Parsimony analysis was carried out on the different datasets in TNT, implementing sectorial search and tree fusion under the following parameters: random addition sequence Wagner builds with 10 000 replications saving 10 trees per replicate and

tree bisection and reconnection (TBR) branch swapping. Branch support was calculated using bootstrap support values (Felsenstein, 1985).

Phylogenetic informativeness and partitioned Bremer support

To measure the contribution of each data partition to the phylogenetic trees from the combined data matrix, phylogenetic informativeness (PI) and partitioned Bremer support (PBS) were assessed. We summed the PI of data partitions based on the tree constructed using the combined data of 13 PCGs and nuclear genes with PhyDesign (López-Giráldez and Townsend, 2011) as a measure of the predicted contribution of a partition to the resolution of the phylogeny. PhyDesign was run on a calibrated Phylobayes tree constructed based on combined data of PCGs and nuclear genes, rooted with *Aeolesthes oenochrous* (AB703463) (see Results). The divergence time of the Phylobayes tree was calculated with the r8s software (Sanderson, 2003) using the age of Chrysomelinae as prior to estimate the age of representative nodes obtained from Gómez-Zurita et al. (2007). The PI was calculated with the Hyphy package (Pond and Muse, 2005) using empirical base frequencies and a time-reversible model of substitution.

The Phylobayes tree from the combined PCGs plus nuclear genes was also chosen to assess the contribution of each gene to the total support of the phylogenetic tree. Average PBS values were calculated using TreeRot v3 (Sorenson and Franzosa, 2007) to generate constrained topologies and Paup v4.0b10 (Swofford, 2002) for finding the best trees under these constraints. The resulting PBS values were then divided by the number of nucleotide sites or amino acids, to obtain an average value for each data partition.

Results

Generation of sequence data

Three nuclear rRNA fragments (*18S*, *28S-D2* and *28S-D3*) and three mitochondrial fragments (*cox1-5'*, *cox1-3'* and *rrnL*) were sequenced with Sanger technology, to produce 963 newly obtained sequences. The nuclear dataset consisted of 340 sequences from 119 taxa and a total of 3008 bp comprising 788 (26.20%) variable sites and 528 (17.55%) parsimony informative sites. The average base composition of nuclear data was 24.0% for A, 25.9% for C, 26.3% for G and 23.7% for T.

Next-generation sequencing was performed for 100 species in 87 genera, including 52 species and 45 genera of Galerucini, and 48 species and 42 genera of Alticini. In addition, representatives of 3 species of other Chrysomeloidea were sequenced as outgroups (Appendix S2) and 7 mitogenomes were obtained from GenBank, for a total 110 mitogenomes, of which 85 were complete with >15 kb in length (15.0-17.7 kb) and 25 mitogenomes were nearly complete at 10-14 kb. All of them include the complete set of 13 PCGs. The gene order of all new sequences followed the presumed ancestral arrangement of the insect mitogenome. All sampled Chrysomeloidea showed the UUU anticodon in tRNA-Lys described by Timmermans et al. (2015) (see Appendix S4) and confirmed this trait as an uncontroverted clade maker of Chrysomeloidea. The total base composition of the PCGs of all analyzed mitogenomes is 32.7% for A, 12.1% for C, 11.9% for G and 43.4% for T on average, for an average AT composition of 76.1%.

Assessment of sequence variation

Length variation of mitochondrial sequences was low and thus produced only minimal alignment ambiguity, which was not investigated further. Plots of pairwise uncorrected sequence divergence against the divergence calculated under a GTR model (Fig. 1) showed that, except for

closely related sequences, the PCGs diverged faster for the transversions than for transitions, as expected from their extreme AT bias of nucleotide composition and thus larger number of sites at which sequences diverge. These differences between transitions and transversions were greatest for fast-diverging markers, such as *nad3*, *atp8* and *cytb*, but less so in slowly diverging genes, such as *cox1* and *cox2*, indicating that above a certain level of divergence, sequences only diverge by further changes in transversions (mostly AT) and their level of saturation is lower. However, at the greatest levels of sequence divergence these plots plateaued, indicating saturation of sequence variation, and again this effect was greatest in the fast-diverging markers (Fig.1). In contrast, for the nuclear genes the divergence of transitions was higher than transversions, and based on this test 18S was not saturated while 28S was mildly saturated. The nonsynonymous substitution rate (K_a) for PCGs was assessed separately for Galerucini and Alticini, using *Anoplophora glabripennis* (Cerambycidae: Lamiinae) as a reference (Appendix S5). Generally, the average K_a value of each gene in Alticini was slightly higher than in Galerucini. The *atp8* gene had the highest substitution rate (average value of Galerucini: 0.43, Alticini: 0.44), followed by the *nad* genes (average value from 0.32 to 0.19), *atp6* (0.10) and the *cox* genes (0.10 to 0.13), while *cox1* had the lowest non-synonymous substitution rate in Galerucini and Alticini. Generally, the higher rates were correlated with lower consistency (e.g. CI=0.153, RI=0.412 in *nad4l* vs. CI=0.131 and RI=0.371 in *atp8*).

Different data types (nuclear genes, PCG, PCG 1st and 2nd codon positions, PCG 3rd codon positions, PCG amino acids) were analyzed with AliGROOVE to evaluate the heterogeneity of sequence variation, by comparing the pairwise sequence divergence of individual terminals with terminals outside of the focal group against the same measure of divergence over the entire data matrix. In general, the mitogenomes had low heterogeneity of sequence composition for most

pairwise comparisons between the sequences of the ingroup, except for the 3rd codon positions (Fig. 2). Likewise, the nuclear genes showed no evidence of compositional heterogeneity in the AliGROOVE test.

Phylogenetic informativeness and Bremer support

For each gene we calculated the Phylogenetic Informativeness (PI) per site along the root-to-tip axis, which measures the power of a given character to resolve polytomies arising from lack of informative character variation. The PI curves for various PCGs were similar in shape, with a steady increase from the root, to a maximum fairly close to the tips at a hierarchical level that defined the genera and their relationships with each other, but then dropped rapidly (Fig. 3). The PI per site was in a similar range for most PCGs, although *nad2*, *nad6* and *atp8* showed substantially higher values. The 3rd codon positions had the highest PI per site along the entire root-to-tip axis. The PI for nuclear *18S rRNA* and *28S-D3 rRNA* genes was extremely low, whereas *28S-D2* was in a similar range as the 2nd positions of the mitochondrial PCGs. *28S-D2* followed a similar curve as the mitochondrial genes with a peak near the tip, while the PI of *18S* and *28S-D3* was generally lower and more uniform. The *nad5* gene showed the highest level of informativeness, followed by the other mitochondrial genes to *atp8* and *nad4l*, which had the lowest level. The net PI of genes mainly was correlated with the length of the gene (Appendix S6).

The total PBS value ranged from 2749 to 23076 for individual PCGs and the average PBS per site ranged from 10.93 to 17.30 of PCGs among all datasets. They were higher than nuclear genes by at least by a factor of 10, whose total PBS ranged from 613 to 1834 for the three gene fragments, and from 0.45 to 0.95 per site (Appendix S7).

Comparison of topologies

We chose 23 key groups to assess the tree topologies obtained with different data combinations

(nuclear, mitogenomic and both combined) and tree construction methods (Phylobayes, MrBayes and parsimony analysis). In general, the trees constructed by each method based on mitogenome data recovered more of the reference nodes than those from the combined nuclear genes (Fig. 4). Six groups (Galerucini, subtribe Luperina, and the *Altica*, *Chabria*, *Chaetocnema* and *Sphaeroderma* groups) were not recovered as monophyletic with the nuclear data, while only two or three of the 23 expected groups were paraphyletic or polyphyletic in the mitogenome trees. In addition, support levels were generally lower in the nuclear and combined-data trees (Table 1), and the nuclear-based topology was more comb-shaped and affected by "rogue taxa" whose positions varied.

The trees constructed with PhyloBayes (Fig. 4) and MrBayes (Appendix S8) had similar topologies for a given data set. The main differences between both topologies were the location of Hylaspina, Galerucina and Oidina. In the MrBayes analysis, the tree based on the combined data of mitogenome and nuclear data was very similar to that from mitogenome data alone, but the former had higher support values. Oidina branched off near the base of Galerucini and recovered the Luperina as two main clades in addition to two species that were in separate positions. The Phylobayes trees based on different data sets showed that the PCGs or PCGs-codon12 data sets recovered the monophyly of all but one of the 23 expected clades, unlike the tree from nuclear genes that recovered only 15 clades. The trees constructed by TNT (Appendix S9) in general were similar to those constructed by the other methods except for the position of Oidina, which in all parsimony trees is placed as the sister group to all other Alticini and Galerucini. Generally the number of reference nodes obtained with parsimony under all data treatments was slightly lower than the ML and Bayesian trees (Table 1). The basal branching of Oidina was also seen in the MrBayes tree using the PCG-codon12 scheme (Appendix S15).

Phylogeny and classification of Galerucini

Most phylogenetic trees (Figs. 5-6; Appendix S10-21) recovered galerucines and alticines ~~were~~ sister groups, with the exception of the parsimony trees (Appendix S18-21) and a single MrBayes tree (Appendix S15) that removed the Oidina from the galerucines. This is clearly contradicted by all nuclear-only trees and thus both galerucines and alticines thus should be treated at an equal taxonomic rank at the level of tribes. In the single case of the PhyloBayes tree on the nuclear genes only, Alticini were paraphyletic, but Oidina remained firmly in the Galerucini (Appendix S10). The subtribes Galerucina, Halyspina and Oidina were each recovered as monophyletic with high support. Luperina was polyphyletic and was split into three subclades corresponding to the Aulacophorina, Dibrotica and Monoleptites groups. Metacyclina always grouped with Galerucina ~~although~~ the available taxon sampling was limited. The placements of the thirteen ~~genera~~ considered 'problematic genera' were stable: four genera (*Lipromela*, *Clitea*, *Sangariola* and *Phygasia*) grouped with Alticini, while the other nine genera (*Laotzeus*, *Mandarella*, *Hespera*, *Taiwanohespera*, *Luperomorpha*, *Decaria*, *Nonarthra*, *Acrocrypta* and *Sphaerometopa*) were nested in Galerucini. The genera *Mandarella*, *Laotzeus*, *Hespera* and *Halticorcus* formed a clade that was always placed together with the Monoleptites group. *Nonarthra* and *Acrocrypta* were grouped with Hylaspina. The relationship of the main clades was: (Dibrotica + (Metacyclina + Galerucina) + (Hylaspina + (Monoleptites + Oidina) + (Luperina + Aulacophorina))).

Discussion

Mitogenomes as effective marker for large-scale phylogenetic inferences

The power of phylogenetic inference depends on many factors, including the kind and number of genes, the density of taxon sampling, the phylogenetic method, the rate and heterogeneity of

character variation, among others. In this study, we used bulk sequencing of total genomic DNA as a cost efficient way to generate a large number of high-quality mitogenome sequences. We then evaluated features of mitogenomes in comparison to another class of widely used (nuclear) genes and tested for the most appropriate likelihood model to improve the tree inference. We find that the mitogenomes of Galerucinae were affected by AT bias, substitution saturation, compositional heterogeneity and rate heterogeneity.

In order to test the contribution of different gene types, phylogenetic informativeness, PBS and topology tests were conducted using the mitogenomes and nuclear genes in side-by-side comparisons. There are many more variable characters in mitogenomes than nuclear rRNA genes, and although we do not have the full-length 28S gene, the potential amount of phylogenetic information in rRNA genes is a lot lower than what is contributed by the full mitogenomes. Each mitochondrial PCG adds a substantial number of characters to the phylogenetic signal, and the power of markers is correlated roughly with the number of characters, as judged by the PBS and PI. The PI per site differs somewhat between genes, which seems to be correlated with the rate of sequence variation (Fig. 3). In order to test the contribution of different gene types, phylogenetic informativeness, PBS and topology tests were conducted using the mitogenomes and nuclear genes in side-by-side comparisons. All analyses indicated that the mitogenome possesses much greater phylogenetic signal to resolve most expected main clades than the nuclear rRNA genes from family and subtribe rank level. Yet, the trees based on combined data sets of nuclear and mitochondrial genes were more effective for deep level phylogenetic analysis, which had higher support value than being used separately.

Various partitioning strategies and phylogenetic tree building methods were used to test the sensitivity of the phylogenetic conclusions to different data treatments. MrBayes searches use GTR

models with fixed number of rate classes (see Appendix S3). The Phylobayes software implements the CAT model, which uses a variable number of rate categories that each are defined by different equilibrium frequencies of nucleotide or amino acid characters estimated from the empirical data (Lartillot and Philippe, 2004). This model is less susceptible to long-branch attraction from compositional and rate bias (Lartillot et al., 2007; Talavera and Vila, 2011; Song et al., 2016). In addition, we removed 3rd codon positions to assess the confounding effects from these fastest evolving markers. The results showed that, unlike trees from all nucleotide positions, the tree based on 1st and 2nd positions did not recover the Galerucini as monophyletic with MrBayes, as Oidina branched at the base of the tree as sister to all other Alticini plus Galerucini, and support values were lower than compared to the tree based on all three codon positions. The curious position of Oidina was also obtained from the mitochondrial data with parsimony, independently of the data treatment. Combined, these two observations indicate that 3rd positions provide valuable phylogenetic signal, and that the erroneous signal leading to this placement of Oidina is not limited to the 3rd positions. This is despite the deviations from uniform variation that were mainly confined to 3rd codon positions in the AliGROOVE analysis (Fig. 2), the high AT bias (see Results) and the saturation of nucleotide change (Fig. 1). The topological differences of the main clades based on the MrBayes and Phylobayes trees are small, and have good agreement between nuclear rRNA and mitogenomes, although rRNA genes alone failed to recover several of these groups. The parsimony analysis also generated a topology that is in overall agreement with the model-based analyses. Overall there is strong consensus in these data, and the information can be extracted equally with the MrBayes and PhyloBayes models, and to some extent even with unweighted parsimony, which suggests that (a) compositional and rate biases evident in mitogenomes do not greatly confound the phylogenetic inferences; (b) mitogenomes have greater power to resolve most expected main clades

than the nuclear rRNA genes from family and subtribe rank level, and this mainly due to the larger number of variable characters, while each site also contains more information than nuclear rRNA genes; and (c) the simpler MrBayes method is sufficient for establishing relationship at subfamily level, which would speed up the phylogenetic analysis of large-scale mitogenomes datasets with the use of simpler algorithms. Yet, the trees based on combined data sets of nuclear and mitochondrial genes were more effective for deep level phylogenetic analysis, which had higher support value than being used separately.

In addition, the scaffold of mitogenomes and rRNA genes produced here allowed a comprehensive phylogenetic analysis of Galerucinae using a data matrix of 273 taxa adding all available data of GenBank based on nuclear genes, PCGs and *rrnL* (Fig. 6). The topology is very similar to the tree from the full PCGs plus nuclear genes. A few of the partial sequences remain difficult to place, such as the 'rogue' lineages BMNH846594 and Exosoma clades whose positions changed among different treatment. However, the analysis reveals an additional advantage of the full mitogenomes, as taxa with limited available data can be integrated into the framework of full-length mitogenomes for the phylogenetic placement of numerous additional taxa with partial sequences, while the overall tree topology is stable.

The origin of the jumping apparatus

There are 40 'problematic genera' whose position is not easily assigned to Galerucini and Alticini based on the absence or presence of the MET (Ge et al., 2012). In this study, 31 species from 13 such genera were included, which showed that *Lipromela*, *Clitea*, *Sangariola* and *Phygasia*, were nested in the Alticini. All of these genera have a spermatheca and aedeagus of the alticine type (Furth and Suzuki, 1994; Furth and Suzuki, 1998). *Laotzeus*, *Mandarella*, *Hespera*, *Taiwanohespera*, *Luperomorpha*, *Decaria*, *Nonarthra*, *Acrocrypta* and *Sphaerometopa* were

grouped in Galerucini with high support, which is consistent with the results of Ge et al. (2011). Those genera have galerucine-type morphological characters, such as slender body shape, pubescent elytra, and an aedeagus without basal spur. *Laotzeus* and *Mandarella* have galerucine-type spermatheca and hind-wing venation. *Hespera* has galerucine-type spermatheca and a single elytral patch typical to Galerucini, but hind-wing venation of the alticine-type. We suggest those genera should be transferred into Galerucini. Also, we corroborate the finding that the jumping apparatus evolved at least twice independently in the main clade of Alticini and probably it was secondarily lost in some genera. This confirmed the conclusion of Ge et al. (2011) who questioned the monophyletic origin of the jumping apparatus assumed in the older literature (Furth and Suzuki, 1994). Thus, the MET should not be the defining character to distinguish Galerucini and Alticini. So when the two tribes are diagnosed, the trait needs to be combined with other characters such as hind wing venation, spermatheca, aedeagus and the number of elytral friction binding patches (two in Alticini, one in Galerucini; unpublished data) that distinguish both groups.

Galerucini-Alticini relationship and the arrangement of Galerucini

The monophyly of the Galerucini and Alticini and their relationships to each other have been contentious and affected the views on their respective rank of subfamily. In this study, the various phylogenetic analyses unanimously support the reciprocal monophyly of both groups, after placing the various 'problematic genera' in either one. As sister groups, they should be treated at equal taxonomic rank. We propose the following system: subfamily Galerucinae includes two tribes Galerucini and Alticini. Consequently, unlike other recent schemes (e.g. Bouchard et al., 2011), we propose to treat groups of genera at a subtribal rank, as follows.

All analysis recovered the subtribes Galerucina+Metacyclina, Hylaspina, Oidina as monophyletic, while Luperina is paraphyletic for the three subtribes Aulacophorina, Dibrotica and

the Monoleptites, which each were monophyletic. Nine 'problematic genera' were included in Galerucini, including *Mandarella*, *Laotzeus*, *Hespera*, *Halticorcus*, which were always grouped with the Monoleptites group, while *Nonarthra* and *Acrocrypta* formed a clade with Hylaspina. The subtribe arrangement should be changed based on the molecular data. In reference to the arrangement of Seeno and Wilcox (1982), a new tribe system is suggested, which includes eight subtribes: Oidina, Galerucina, Hylaspina, Metacyclina, Luperina, Aulacophorina, Diabroticina and Monoleptina, of which the latter three were previously considered of suprageneric or section rank within Luperini. Only the monotypic subtribe Decarthrocerina proposed by Laboissière (1937) from Algeria, which has been moved between Alticinae (Seen and Wilcox, 1982) and Galerucinae (Beenen, 2010; Bouchard et al., 2011), was not available for molecular analysis and thus remains *incertae sedis* at the tribal level.

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Table 1 Topological comparisons of MrBayes trees, PhyloBayes trees and parsimony trees, based on different data partition

Nodes	Nuclear genes			13PCGs-by gene			13PCGs_codon12			13PCG-AA			Nuclear genes+13PCGs		
	B	P	MP	B	P	MP	B	P	MP	B	P	MP	B	P	MP
1	M/0.91	Pa	Pa	M/1	M/1	M	Pa	M/0.99	M	--	M/0.99	--	M/1	M/1	M
2	M/1	M/0.3	M	M/0.61	M/1	Pa	M/1	M/1	Pa	--	M/1	--	M/1	M/1	Pa
3	Pa	Pa	Pa	M/0.5	M/0.94	Pa	M/1	M/0.91	M	--	M/0.80	--	M/1	M/0.88	M
4	M/1	M/0.99	M/0.51	M/1	M/1	M/1	M/1	M/1	M/1	--	M/1	--	M/1	M/1	M/0.98
5	M/0.61	M/0.89	Pa	M/1	M/1	M/0.99	M/1	M/1	M/0.9	--	M/1	--	M/1	M/0.99	M/0.5
6	Pa	Pa	Pa	Pa	M/0.94	M	M/0.7	M/0.99	M	--	P	--	M/1	M/0.99	Pa
7	Pa	Pa	Pa	M/1	M/0.99	M	M/1	M/1	M	--	M/0.99	--	M/0.99	M/0.99	M
8	Pa	M/1	Pa	M/1	M/1	M/1	M/0.9	M/1	M	--	Pa	--	M/1	M/1	M/1
9	M/1	M/1	M/1	M/1	M/1	M/1	M/1	M/1	M/1	--	M/1	--	M/1	M/1	M/1
10	M	M/0.75	Pa	M/1	M/1	M/0.65	M/1	M/0.99	M/0.67	--	M/1	--	Pa	M/0.56	M
11	M/1	M/1	Pa	M/1	M/1	M/1	M/1	M/1	M/1	--	M/1	--	M/1	M/1	M/1
12	M/1	M/1	M/1	M/1	M/1	M/1	M/1	M/1	M/1	--	M/0.99	--	M/1	M/1	M/1
13	M/1	M/1	M/1	M/1	M/1	M/1	M/1	M/1	M/1	--	M/1	--	M/1	M/1	M/1
14	Pa	Pa	Pa	M/1	M/0.95	M	M/0.8	M/0.93	Pa	--	M/0.99	--	M/1	M/0.97	Pa
15	M/1	M/1	M/0.95	M/0.99	M/0.99	M/0.83	M/1	M/0.99	M/0.53	--	M/0.99	--	M/1	M/1	M/0.87
16	M/1	M/1	M/1	M/1	M/1	M/0.98	M/1	M/1	M/0.99	--	M/1	--	M/1	M/1	M/0.98
17	M/1	M/0.99	M/0.73	M/0.81	M/1	M/0.55	M/1	M/1	M/0.50	--	M/1	--	M/1	M/1	M/0.87
18	M/0.94	M/0.89	M	M/0.5	M/1	M	M/1	M/0.51	M	--	M/0.67	--	M/0.6	M/1	M
19	M/1	M/1	M	M/0.58	M/1	M	M/1	M/1	Pa	--	M/1	--	M/1	M/1	Pa
20	Pa	Pa	Pa	Pa	Pa	Pa	Pa	Pa	Pa	--	Pa	--	Pa	Pa	Pa
21	M/1	M/1	M/0.99	M/1	M/1	M/1	M/1	M/1	M/1	--	M/1	--	M/1	M/1	M/0.94
22	M/0.97	M/0.97	Pa	M/0.83	M/0.99	M	M/1	M/0.93	M	--	M/1	--	M/1	M/1	M/0.5
23	M/1	M/1	M/1	M/1	M/1	M1	M/1	M/1	M/1	--	M/1	--	M/1	M/1	M/0.95
Total	16	17	12	21	22	20	21	22	19		20		21	22	18

Note: B=MrBayes trees; P=Phylobayes trees; MP: Maximum parsimony; M=monophyly; Pa=Paraphyly. 1=Alticini; 2=Galerucini; 3=Altica group; 4=Blepharida group; 5= Blepharida group + Nisotra group; 6=Chabria group; 7=Chaetocnema group; 8=Griva group; 9=Lanka group; 10=Longitarsus group; 11=Nisotra group; 12=Oedionychus group; 13=Phygasia group; 14=Spheroderma group;

15=Aulacophorina; 16=Diabroticina; 17=Galerucina+Metacyclina; 18=Hylaspina; 19=Hylaspina+Nonarthra; 20=Luperina; 21=Monoleptites; 22= Monoleptites + four ‘problematic genera’; 23=Oidina.

FIGURE CAPTIONS

Fig. 1. Saturation plots for nucleotide datasets of PCGs and a nuclear gene performed in DAMBE5 with the GTR model selected as a reference model. S: transition rate; V: transversion rate.

Fig. 2. Heterogeneous sequence divergence of mitochondrial genomes for different data sets. The mean similarity score between sequences is represented by a colored square. The scores are ranging from -1 indicating full random similarity, to +1 indicating non-random similarity, which is visualized by a color range from dark blue (-1) to bright orange (+1). Blue indicated the opposite. All taxa names were listed on top and the right hand side of the matrix with different color, black (outgroup), blue (Alticini) and red (Galerucini).

Fig. 3. The phylogenetic informativeness per site in 13 PCGs and nuclear genes. The tree topology is from the Phylobayes analysis of the combined data set, with divergence times estimated with the R8s software.

Fig. 4. The topological comparison of Phylobayes trees constructed based on different data partitions and treatments.

Fig. 5. Phylobayes tree based on the combined data of 13 PCGs and nuclear genes (118 taxa). Numbers above each node are posterior probabilities.

Fig. 6. Phylobayes tree constructed using the combined mitogenome dataset and additional data from GenBank (273 taxa). Numbers above each node are posterior probabilities.

Supporting Information

Additional supporting information can be found in the online version of this article:

Appendix S1. Primers used for PCR.

Appendix S2. Samples and register gene information.

Appendix S3. Nucleotide substitution model chosen of each marker by jModelTest.

Appendix S4. The alignment results of tRNAs of Chrysomeloidea. All the anticodon are UUU (highlighted by black square).

Appendix S5. Nucleotide substitution rates between Galerucini and Alticini among 13 PCGs. Ka was calculated in a pairwise fashion, using *Anoplophora glabripennis* as a reference.

Appendix S6. The net phylogenetic informativeness of 13 PCGs, Codon1, 2, 3 and nuclear genes (18S, 28S-D2, 28S-D3). The tree was constructed by Phylobayes software based on the combined data set of 13 PCGs and nuclear genes, with divergence time by R8s software.

Appendix S7. The average PBS (a) and average PBS per site value (b) of different genes to a trees based on 13 PCGs and nuclear genes.

Appendix S8. The topological comparison of Bayesian trees based on different data partition.

Appendix S9. The topological comparison of MP trees based on different data partition.

Appendix S10. Phylobayes tree based on combined data of nuclear genes (18S rRNA, 28S-D2 rRNA, and 28S-D3 rRNA). Numbers above each node are posterior probabilities.

Appendix S11. Phylobayes tree based on combined data of 13 PCGs. Numbers above each node are posterior probabilities.

Appendix S12. Phylobayes tree based on combined data of 13 PCGs_codon12. Numbers above each node are posterior probabilities.

Appendix S13. Phylobayes tree based on combined data of 13 PCGs_AA. Numbers above each node are posterior probabilities.

Appendix S14. Bayesian tree based on combined data of nuclear genes (18S rRNA, 28S-D2 rRNA, and 28S-D3 rRNA). Numbers above each node are posterior probabilities.

Appendix S15. Bayesian tree based on combined data of 13 PCGs-codon12. Numbers above each

node are posterior probabilities.

Appendix S16. Bayesian tree based on combined data of 13 PCGs. Numbers above each node are posterior probabilities.

Appendix S17. Bayesian tree based on combined data of 13 PCGs of mitochondrial genomes and nuclear genes (118 taxa). Numbers above each node are posterior probabilities.

Appendix S18. Strict consensus tree of parsimony analysis based on combined data of nuclear genes (18S rRNA, 28S-D2 rRNA, and 28S-D3 rRNA). Numbers above each node are bootstrap support.

Appendix S19. Strict consensus tree of parsimony analysis based on combined data of 13 PCGs-codon12. Numbers above each node are bootstrap support.

Appendix S20. Strict consensus tree of parsimony analysis based on combined data of 13 PCGs. Numbers above each node are bootstrap support.

Appendix S21. Strict consensus tree of parsimony analysis based on combined data of 13 PCGs of mitochondrial genomes and nuclear genes (118 taxa). Numbers above each node are bootstrap support.

Appendix S22. Data matrix of 13 PCGs in nexus format.

Appendix S23. Data matrix of nuclear genes in nexus format.

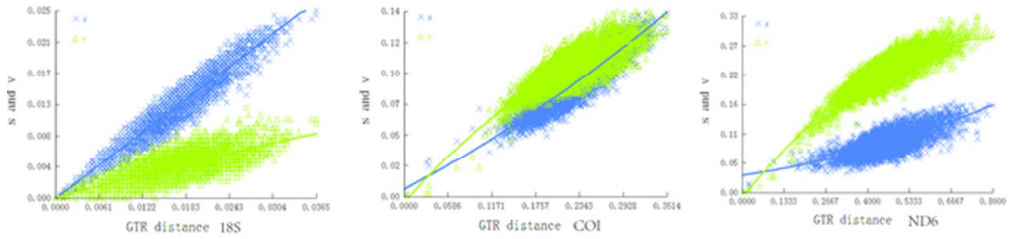

Fig. 1. Saturation plots for nucleotide datasets of PCGs and a nuclear gene performed in DAMBE5 with the GTR model selected as a reference model. S: transition rate; V: transversion rate.

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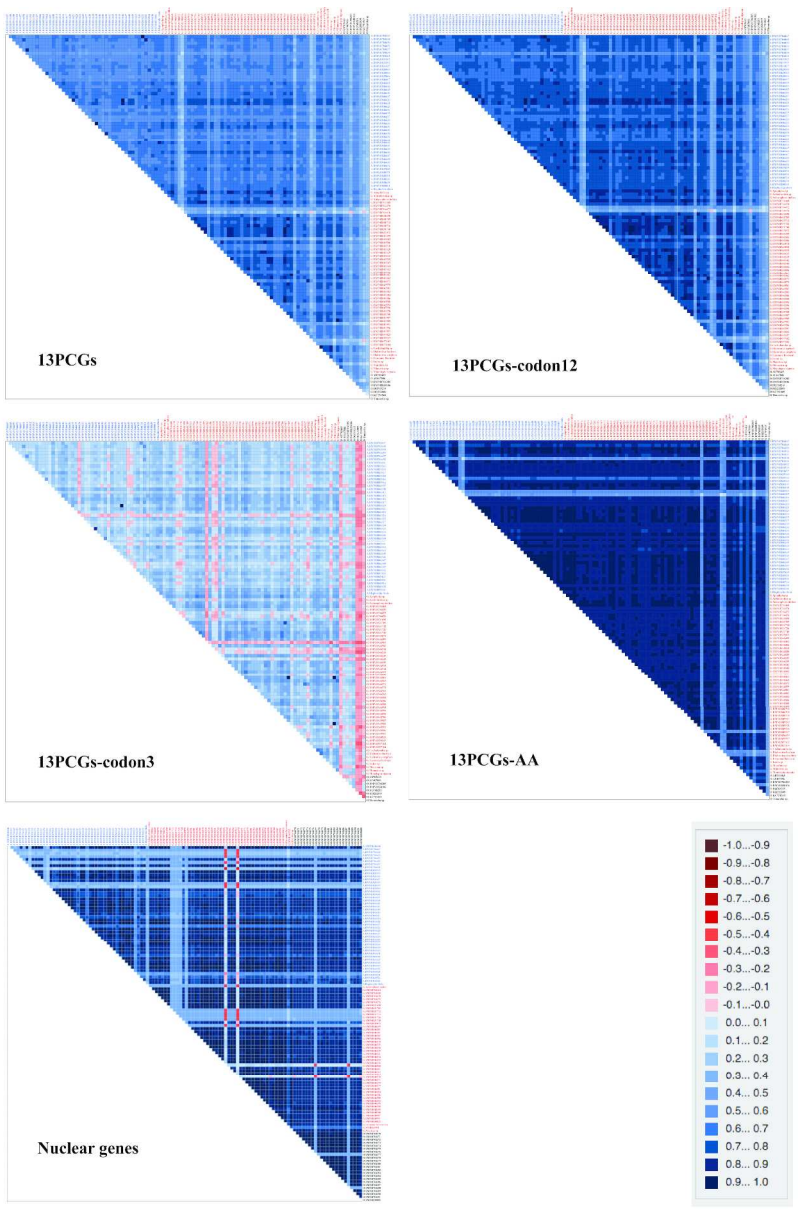

Fig. 2. Heterogeneous sequence divergence of mitochondrial genomes for different data sets. The mean similarity score between sequences is represented by a colored square. The scores are ranging from -1 indicating full random similarity, to +1 indicating non-random similarity, which is visualized by a color range from dark blue (-1) to bright orange (+1). Blue indicated the opposite. All taxa names were listed on top and the right hand side of the matrix with different color, black (outgroup), blue (Alticini) and red (Galerucini).

232x348mm (300 x 300 DPI)

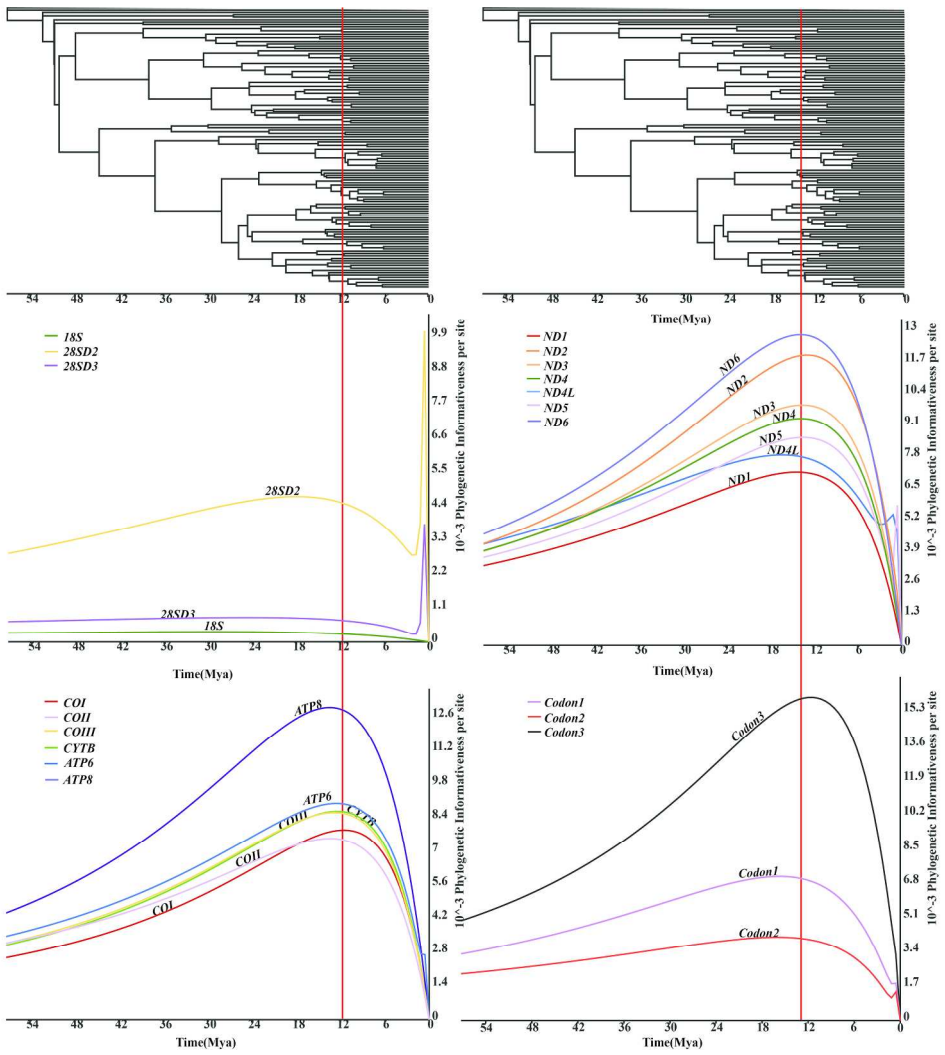

Fig. 3. The phylogenetic informativeness per site in 13 PCGs and nuclear genes. The tree topology is from the Phylobayes analysis of the combined data set, with divergence times estimated with the R8s software.

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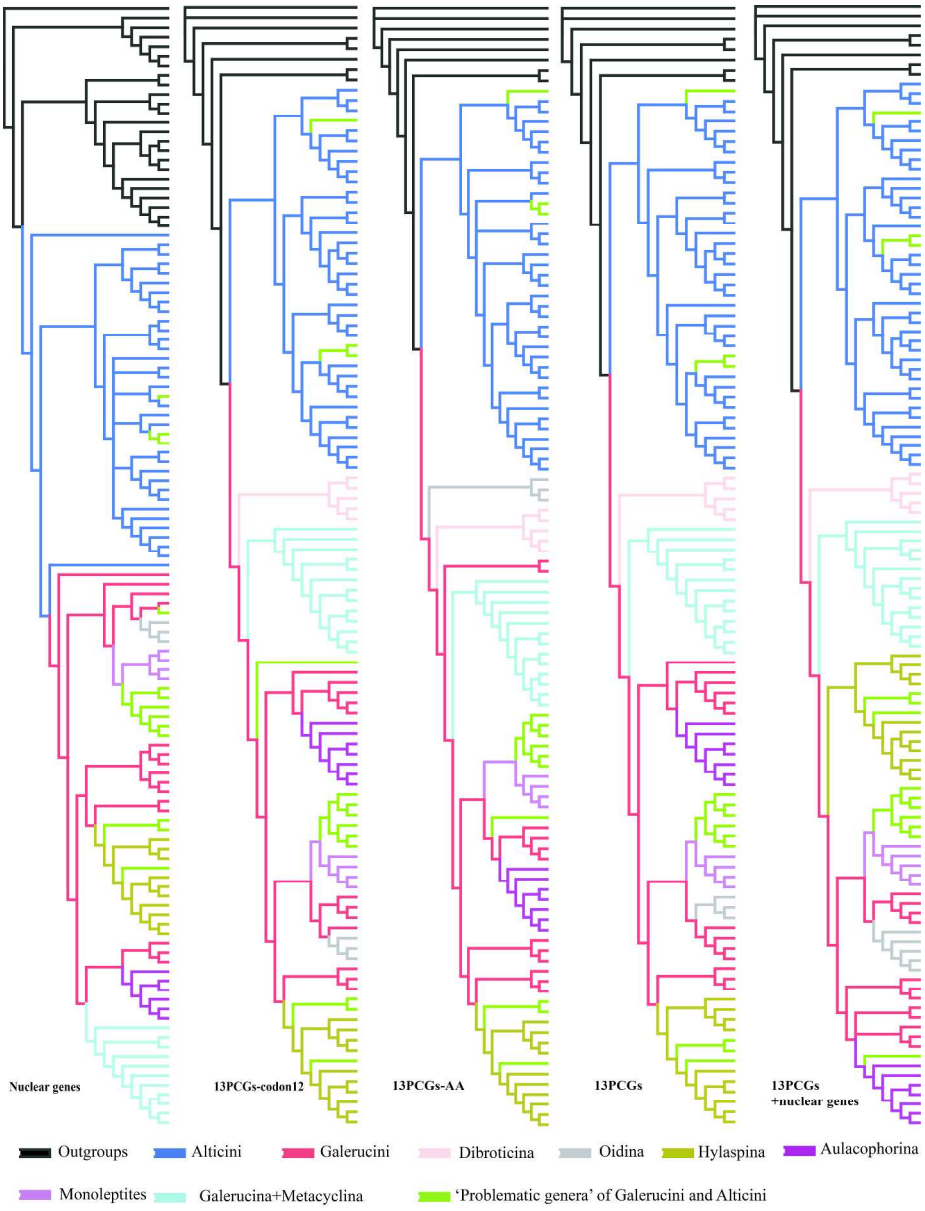

Fig. 4. The topological comparison of Phylobayes trees constructed based on different data partitions and treatments.

307x402mm (300 x 300 DPI)

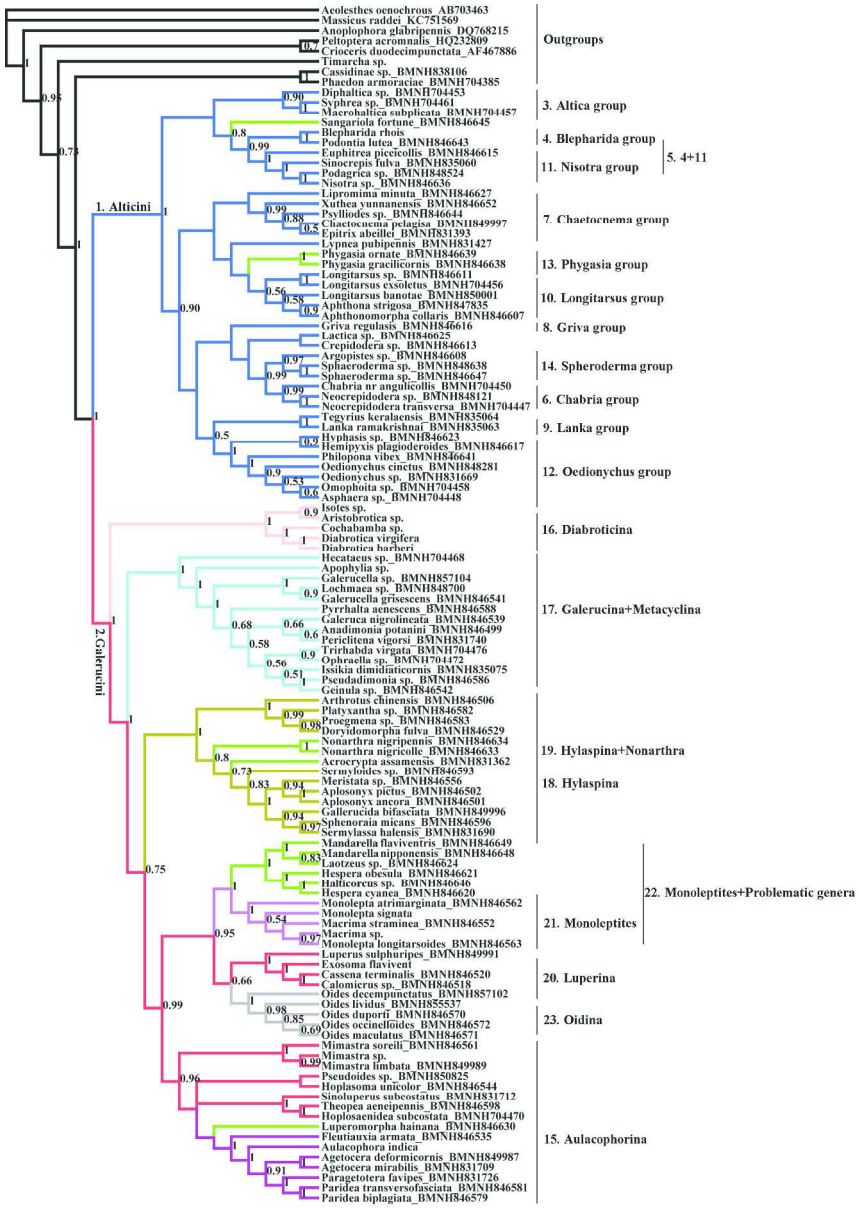

Fig. 5. Phylobayes tree based on the combined data of 13 PCGs and nuclear genes (118 taxa). Numbers above each node are posterior probabilities.

228x294mm (300 x 300 DPI)

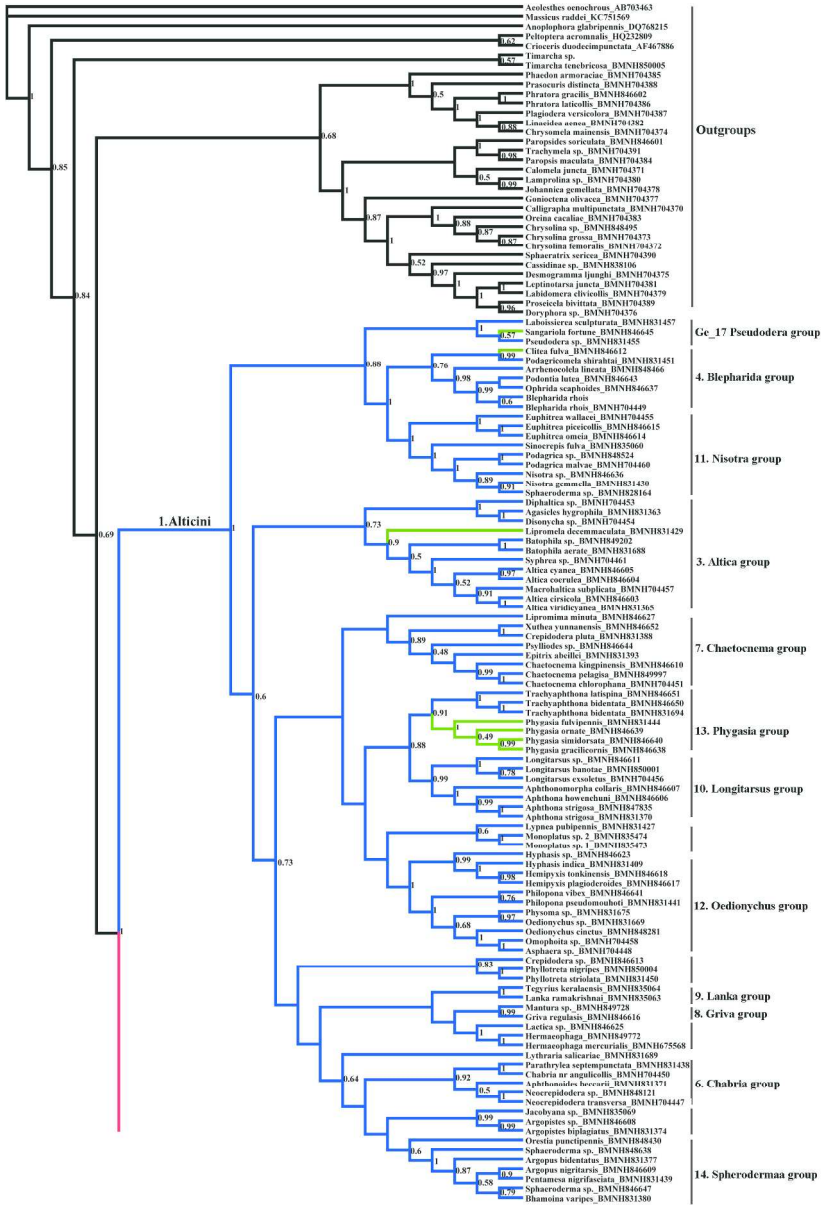

Fig. 6. Phylobayes tree constructed using the combined mitogenome dataset and additional data from GenBank (273 taxa). Numbers above each node are posterior probabilities.

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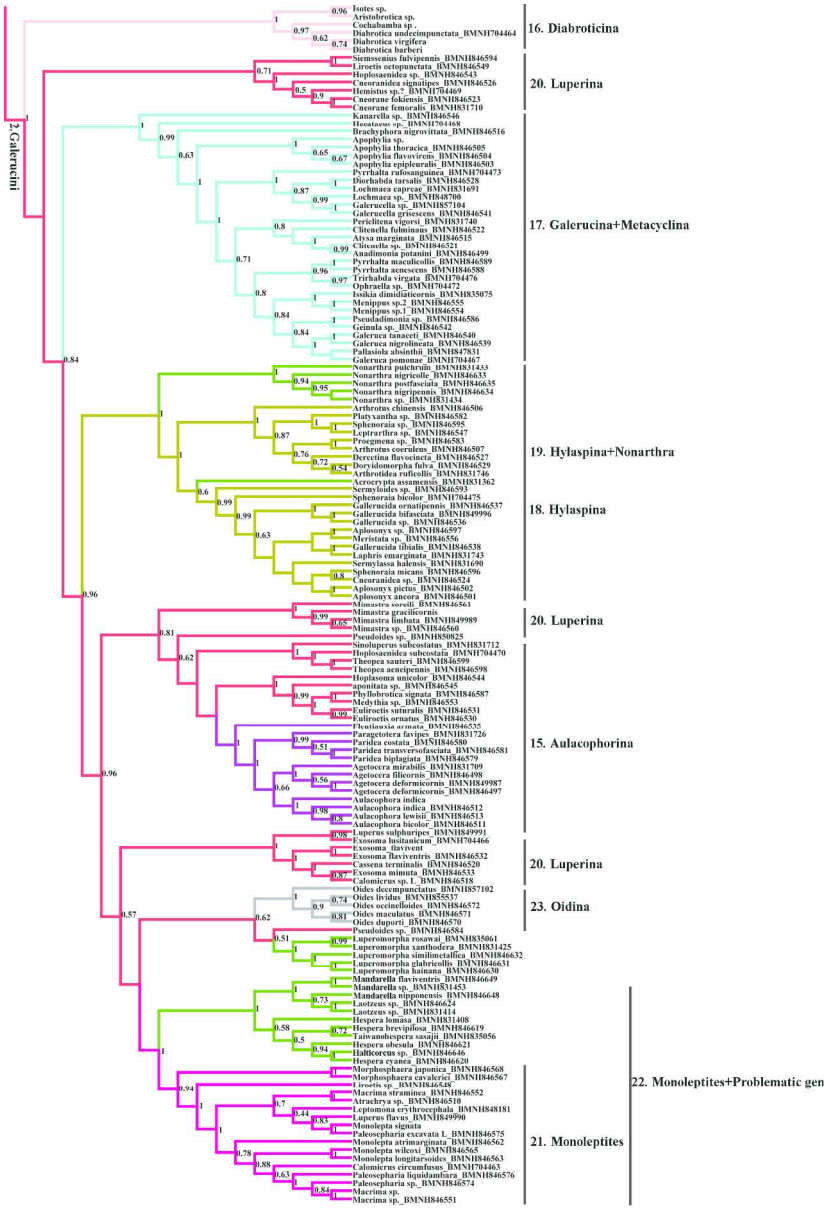

Fig. 6. Phylobayes tree constructed using the combined mitogenome dataset and additional data from GenBank (273 taxa). Numbers above each node are posterior probabilities.

199x293mm (300 x 300 DPI)